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Cimetidine inhibits in vivo growth of human colon cancer and reverses histamine stimulated in vitro and in vivo growth

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Abstract

The effect of histamine and cimetidine on the growth of four human colon cancer cell lines was studied. Histamine significantly stimulated the uptake of tritiated thymidine in vitro in a dose dependent manner, to a maximum of 120% and 116% of controls for C170 and LIM2412, respectively. This effect was antagonised by cimetidine, but not diphenhydramine. Histamine also stimulated a dose dependent increase in cyclic adenosine monophosphate accumulation in C170 cells, antagonised by cimetidine. When grown as subcutaneous xenografts in Balb/c *nu/nu* mice, cimetidine had a significant inhibitory effect on the same two cell lines. The final volume of C170 tumours in animals given cimetidine was 44% of controls. This response was dose dependent, plateauing at a cimetidine dose of 50 mg/kg/day. The final volume of LIM2412 tumours in animals given cimetidine was 60% of controls. Histamine administered locally by a mini-osmotic pump stimulated C170 tumour growth to 164% of controls, was antagonised by cimetidine at a dose of 200 mg/kg/day, but not by lower concentrations. Histamine has a trophic effect on at least two colorectal cancer cell lines in vivo and in vitro. As this effect is antagonised by cimetidine, it may be mediated via tumour histamine type 2 receptors.

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Cimetidine has been shown to inhibit the growth of several types of tumours in animals[1–4] and to induce tumour regression in man.[5] One controlled trial in gastric cancer has shown a significant survival advantage in patients who received postoperative cimetidine.[6] These effects were largely attributed to the important effects of cimetidine on the immune function.

Our recent finding that cimetidine inhibited the growth of carcinogen induced colonic tumours in rats, with corresponding reductions in the cellular proliferative indices,[7] led us to investigate further the role of histamine in colonic cancer. The present study examines the effect of histamine and cimetidine on colorectal cancer cell lines to determine the presence of a direct non-immunological effect in this type of malignancy.

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Methods

MATERIALS

Four human colonic adenocarcinoma cancer cell lines were studied. C170[8] and LoVo[9] were gifted by CRC Laboratories, Nottingham, UK and LIM2412 and LIM2405[10] were gifts from the Ludwig Institute, Melbourne, Australia. All cell lines were maintained in RPMI plus 10% fetal calf serum (FCS) (Cytosystems Pty Ltd, Australia) at 37°C in 5% CO_2.

Chemicals used were: cimetidine (Smithkline Beecham, Australia); diphenhydramine (Aldrich Pty Ltd, USA); isobutylmethylxanthine (IBMX), thymidine, and histamine hydrochloride (Sigma Chemical Co, St Louis, USA); ^{3}H-methylthymidine (Dupont, USA) and; Hams F12/MEM media (Cytosystems Pty Ltd, Australia).

CELL PROLIFERATION ASSAY

Tumour cells were suspended at a concentration of 1×10^5 cells/ml in RPMI-1640 with 10% FCS, distributed onto a 96 well microtitre plate and incubated at 37°C for 24 hours. The cells were then synchronised by a further 24 hours' incubation in a 0·6 mmol/l solution of thymidine in serum free RPMI. The supernatant was then replaced with Hams F12/MEM media containing the test drugs (histamine, diphenhydramine, and cimetidine) at a range of concentrations (1×10^{-10} M to 1×10^{-6} M) in a volume of 200 µl. Control wells contained media alone. After the addition of 50 µl of 0·1 µCi of ^{3}H-methylthymidine, the cells were incubated for 24 hours. After this, they were harvested, washed, and counted using a Packard scintillation beta counter (model B4430) using a DPM option (modified from Kusyk *et al* 1986[11]).

Each concentration was tested in quadruplicate and repeated on three separate occasions. Results are expressed as a mean (SEM) percentage of the control. A one way analysis of variance (ANOVA) was used to compare treated with control wells.

QUANTIFICATION OF STIMULATED INTRACELLULAR CYCLIC ADENOSINE MONOPHOSPHATE (cAMP)

C170 cells were suspended in serum free RPMI-1640 containing 0·5 mM IBMX at a concentration of $1{\cdot}25\times10^5$ cells/250 µl. Aliquots (125 µl) of histamine were added in a

range of concentrations from 1×10^{-7} M to 1×10^{-3} M, with or without the addition of a histamine antagonist at a concentration of 1×10^{-4} M. The cells were then incubated for 10 minutes at 37°C.[12] The incubation was terminated by the addition of 0·5 ml of 0·001 M HCl in chilled ethanol to fix the cells and allow for cAMP extraction. The suspension was mixed, then centrifuged for 15 minutes at 15 000 rpm. Some 0·4 ml of supernatant was drawn off and the contents were extracted using a Speed Vac Concentrator (Savant Instruments, NY, USA).

After reconstitution in 2 ml of the buffer solution provided, the concentration of cAMP present was measured using a monoclonal antibody based kit (Amersham, UK). Results were expressed in fmol of cAMP/10^5 cells, after corrections were made for the dilutions performed during the assay. Each drug concentration was measured in triplicate and repeated in two experiments. The data were non-parametric and were analysed using a Kruskal-Wallis test.

XENOGRAFTS

Six to 10 week old male Balb/c *nu/nu* mice (ANSTO, Lucas Heights, Australia) were used as tumour hosts. Four separate experiments were carried out.

In the first experiment, 1 mm^3 fragments of tumours developed from each cell line were subcutaneously implanted into anaesthetised animals (Hypnorm, Janssen Pharmaceutica, Australia). The mice were then randomly allocated to either treatment or control groups of 10 animals for each cell line. Control animals had free access to autoclaved deionised drinking water. For the animals in the treatment group, the water contained the histamine type 2 receptor antagonist, cimetidine, at a concentration of 0·4 mg/ml. As nude mice have previously been shown to drink an average of 5 (0·6) ml/day[2] (as confirmed in our own laboratory), this would produce an oral intake of cimetidine that approximate to 100 mg/kg/day.[2] Treatment began on the day of tumour implantation. The experiment ended when the tumours began to ulcerate, usually between the 21st and 28th days. The drinking water was renewed every three days until the end of the experiment.[2]

After implantation, most xenografts grew into single spherical or ovoid tumours. The two greatest tumour perpendicular diameters were measured three times weekly with vernier callipers, and their volumes (V) were calculated by the formula V=0·5 length×(width).[2 13] In a few cases the tumours became bosselated, with two or more foci of tumour growth. In these cases each ovoid component of the tumour was measured separately, using the method described above, and the total volume was calculated from their sum. Those animals which failed to develop a tumour mass were excluded from analysis.

At the end of the experiment, all mice were killed by intraperitoneal dose of pentabarbitone sodium (Lethobarb, Virbas Pty Ltd, Australia). Tumours were harvested and fixed in 10% formalin for histological examination.

In the second experiment, xenografts were produced by a subcutaneous injection in the left flank of each animal of 1×10^6 C170 cells suspended in 100 µl of RPMI-1640. Immediately after this injection, the animals were randomised to treatment

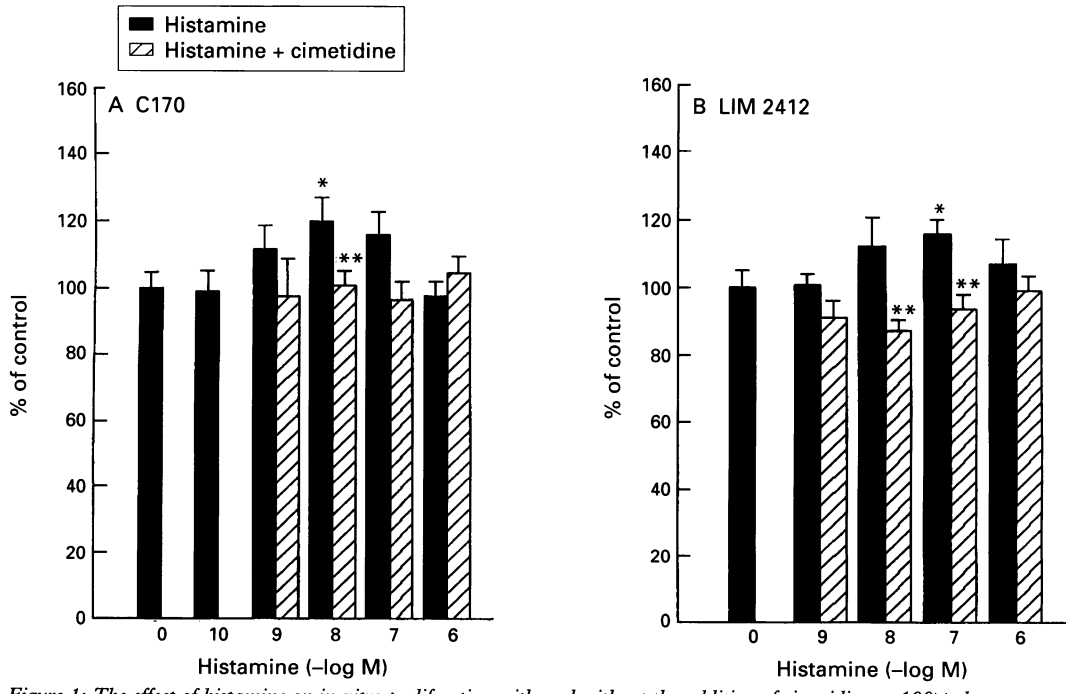

Figure 1: The effect of histamine on in vitro proliferation with and without the addition of cimetidine at 100× the histamine concentration. Combined results from three experiments performed in quadruplicate. Results are expressed as a percentage of mean ^{3}H-methyl thymidine uptake relative to controls and were compared using ANOVA. Error bars represent the SEM. Those marked with a single asterisk were significantly greater than controls ($p<0·05$), those marked with a double asterisk were significantly less than those treated with histamine alone. (A) Cell line C170: (B) Cell line LIM2412.

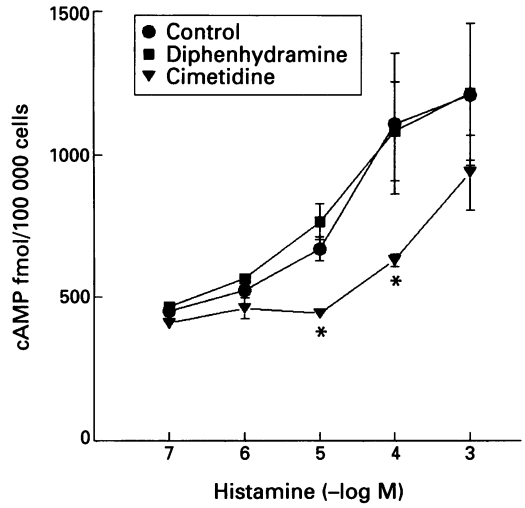

*Figure 2: The effect of histamine on intracellular levels of cyclic adenosine monophosphate cAMP in C170 cells, with and without the addition of cimetidine at a concentration of 10^{-4}M. Combined results from two experiments performed in triplicate. Results are expressed as mean fmol cAMP/10^5 cells and error bars represent the SEM. Raw data were compared using a Kruskal-Wallis test. *$p<0.05$.*

groups receiving cimetidine in the drinking water at concentrations of 0, 10, 25, or 50 mg/kg/day. The experiment was then repeated with cimetidine concentrations of 0, 50, 100, and 200 mg/kg/day and was otherwise identical to the first experiment.

In the third experiment, each animal had a 14 day mini-osmotic pump (Model 2002, Alza Corporation, Palo Alto, USA) implanted subcutaneously in the left flank under anaesthesia. A suspension of 1×10^6 C170 cells in 100 µl of RPMI-1640 was then injected subcutaneously in the region of the pump's orifice. In one group of 10 animals, the pump contained 0·9% phosphate buffered saline (PBS) and the mice received no cimetidine. In the remaining three groups of 10 animals, the pump contained histamine at a concentration of 1×10^{-2} M in PBS, delivered at a rate of 1×10^{-10} mol/min. One group received no cimetidine in the drinking water, one group received cimetidine at a rate of 50 mg/kg/day and another cimetidine at a rate of 200 mg/kg/day. All pumps were replaced after 14 days. The experiment was otherwise identical to experiments 1 and 2.

In the fourth experiment tumours were inoculated as in experiment 2. Animals were randomly allocated to act as controls or to receive the histamine type 1 receptor antagonist, diphenhydramine, in the drinking water at concentrations of 0·02 mg/ml or 0·08 mg/ml, approximating to daily doses of 5 mg/kg and 20 mg/kg respectively. Concentrations of diphenhydramine of 50 mg/kg/day and 100 mg/kg/day had previously been found in our laboratory to be toxic in these animals. There were 10 animals in each group and the experiment proceeded as for experiments 1 and 2.

In each case the data from treated and untreated groups were compared using a Kruskal-Wallis test.

Results

CELL PROLIFERATION ASSAY

Histamine produced a dose dependent stimulation of cell proliferation in the cell lines C170 (Fig 1A) and LIM2412 (Fig 1B). A bell shaped curve of response to histamine was seen, with maximal stimulation occurring at histamine concentrations of 1×10^{-8} M and 1×10^{-7} M for C170 and LIM2412 respectively. For both these cell lines the stimulatory effect of histamine was significantly antagonised in the presence of 100× molar excess of cimetidine. The addition of a 100× excess of diphenhydramine did not alter the response to histamine of either of these cell lines (data

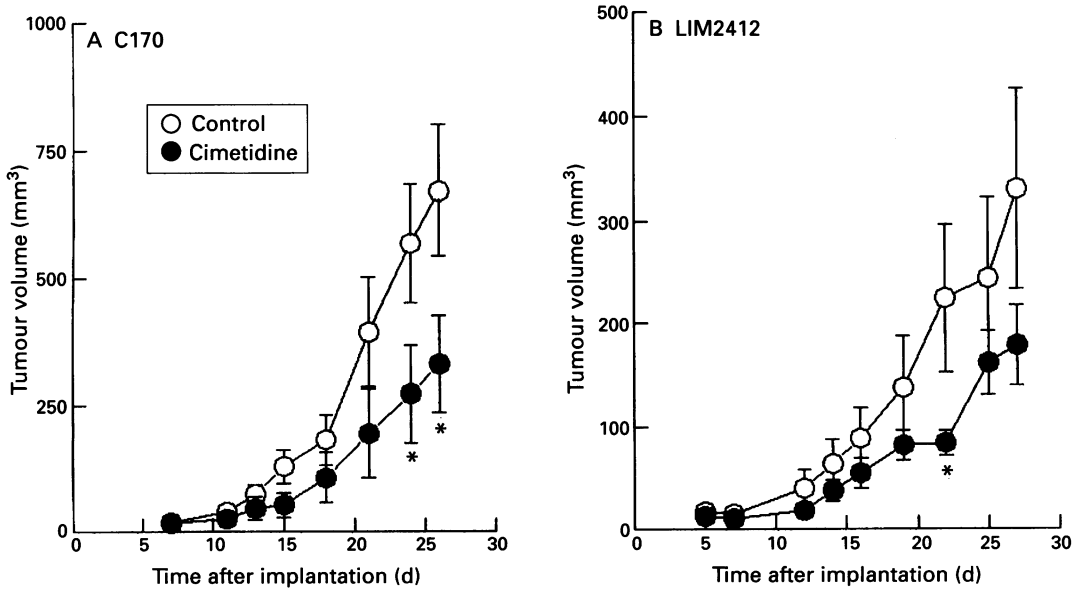

Figure 3: The growth of human colon cancer xenografts implanted subcutaneously in Balb/c nu/nu mice. Treated animals received cimetidine in the drinking water at a dose of 100 mg/kg/day. Points represent mean tumour volumes, error bars represent the SEM. Tumour volumes were compared using a Kruskal-Wallis test; those marked with an asterisk were significantly less than controls ($p<0.05$). (A) Cell line C170 xenografts: control $n=6$; cimetidine $n=8$. (B) Cell line LIM2412 xenografts: control $n=5$; cimetidine $n=7$.

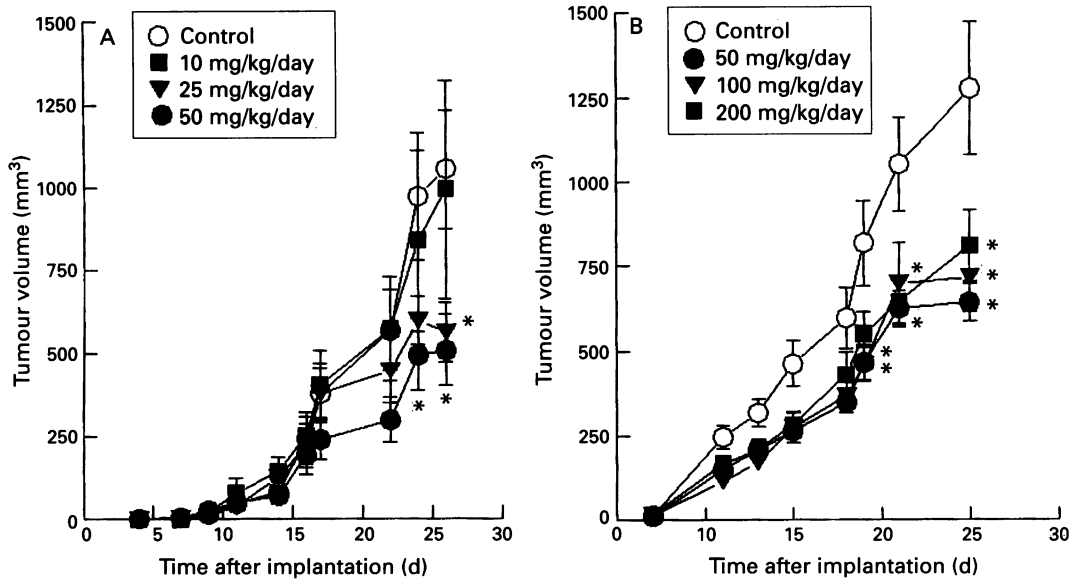

Figure 4: The effect of a range of cimetidine doses on the growth of C170 xenografts. Points represent mean tumour volumes, error bars represent the SEM. Tumour volumes were compared using a Kruskal-Wallis test; those marked with an asterisk were significantly less than controls ($p<0.05$). (A) Control n=8; cimetidine 50 mg/kg/day n=8; cimetidine 25 mg/kg/day n=7; cimetidine 10 mg/kg/day n=8. (B) Control n=21; cimetidine 200 mg/kg/day n=15, cimetidine 100 mg/kg/day n=16; cimetidine 50 mg/kg/day n=16.

not shown). Cimetidine did not effect the basal rate of proliferation of either of these cell lines in the absence of histamine (data not shown).

Neither histamine nor cimetidine had any significant effect on the cell lines LIM2405 or LoVo (data not shown).

QUANTIFICATION OF STIMULATED INTRACELLULAR CAMP

Histamine stimulated a dose dependent increase in intracellular cAMP production in C170 cells to a maximum of 275% of basal levels (Fig 2). This effect was antagonised by cimetidine but not by diphenhydramine.

XENOGRAFTS

No animal developed macroscopic deposits of metastatic disease in the lungs, liver, or other organs. Final animal body weights did not significantly differ between control animals and those receiving either cimetidine or histamine and neither did the proportion of animals in which tumours were successfully induced.

In the first experiment, the growth of both C170 and LIM2412 tumours was significantly inhibited by cimetidine at a dose of 100 mg/kg/day, with reductions in the final mean tumour volumes to 43·5% and 59·5% of controls, respectively (Fig 3). The remaining two cell lines, LIM2405 and LoVo, were not significantly effected (data not shown).

The effect of cimetidine on C170 tumour growth was dose dependent, plateauing at a dose of 50 mg/kg/day (Fig 4).

Diphenhydramine, at doses of 5 mg/kg/day and 20 mg/kg/day, did not significantly effect tumour growth (data not shown).

Exogenous histamine produced an increase in the final mean tumour volume to 163·6% of control (Fig 5). This effect was completely reversed by cimetidine at a dose of 200 mg/kg/day, but not at 50 mg/kg/day.

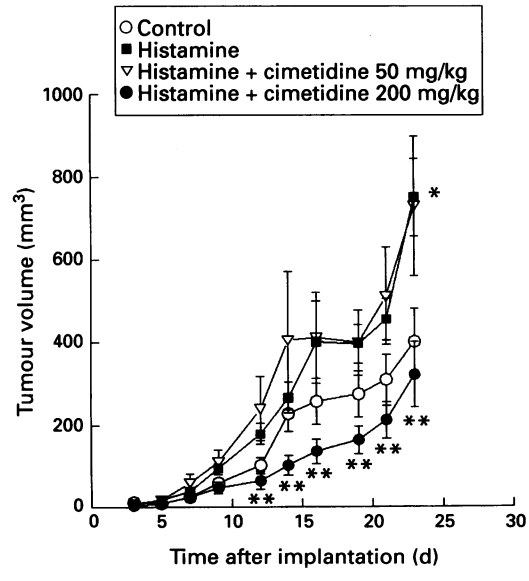

Figure 5: The effect of exogenous histamine delivered at a rate of 10^{-10} mol/min on C170 xenograft growth, with and without treatment with oral cimetidine. Points represent mean tumour volumes, error bars represent the SEM. Tumour volumes were compared using a Kruskal-Wallis test. Tumours marked with a single asterisk were significantly greater than controls. Tumours marked with a double asterisk were significantly less than those in animals receiving histamine without cimetidine ($p<0.05$). Control n=8; subcutaneous histamine 1×10^{-10} mol/min n=9; subcutaneous histamine plus cimetidine 200 mg/kg/day n=10; subcutaneous histamine plus cimetidine 50 mg/kg/day n=8.

TUMOUR HISTOLOGY

Histological sections of resected tumours of each cell line showed poorly differentiated tumours with little surrounding cellular inflammatory reaction in the host tissues. Larger tumours exhibited central necrosis. There was no macroscopic difference between tumours from treated and untreated animals.

Discussion

These experiments have shown that cimetidine has an inhibitory effect on both in vivo and in vitro growth of two or four human colon cancer cell lines tested.

We have already shown that cimetidine inhibits the growth of carcinogen induced colorectal cancer.[7] Transient in vivo inhibition

of xenografts of two colon cancer cell lines, has also been described previously.[14] In addition, cimetidine has been found to have an inhibitory effect on several other tumour types[1-4] and has induced a clinical response in human cancers.[5 6] It has been thought that cimetidine acts through immunostimulation[1-4 15-17] mediated via histamine receptors on lymphocyte suppressor cells;[18 19] however, the results of our experiments in vitro suggest that a direct inhibitory effect on tumour cell proliferation is also present, for at least some tumours.

The fact that histamine stimulation of in vitro growth for both C170 and LIM2412 is antagonised by cimetidine, but not diphenhydramine, suggests that this is an H_2 receptor phenomenon. This is confirmed by histamine's stimulation of intracellular cAMP production in C170 cells, again antagonised by cimetidine, as these receptors are known to be linked to adenylate cyclase.[20] Functional H_2 receptors have been reported on other tumour types, including gastric cancer[21] and melanoma.[22]

A recent paper by Watson *et al*[23] found that, in contrast to our own results, histamine did not stimulate the in vitro proliferation of C170, but did stimulate the growth of the gastric cancer cell line, MKN45, both in vivo and in vitro. Histamine was found to stimulate intracellular cAMP production in MKN45, but not C170, suggesting the presence of tumour H_2 receptors on the former cell line as the mechanism for histamine's action. The effect of histamine on the rate of cellular proliferation of C170 in our model was modest, as was the volume of production of cAMP. The difference in findings between our groups may represent differences in cell characteristics that have arisen after a period of separate evolution in different laboratories, as well as differences in assay technique. It would be interesting to see the effect of cimetidine treatment of xenografts on Watson's C170 cells, as this experiment was not reported to have been performed.

The histamine pump experiments on our C170 xenografts showed a dose effect of histamine in vivo. While exogenous histamine stimulated basal tumour growth, it also led to an increase in the required dose of cimetidine needed to inhibit tumour growth. This suggests a direct antagonism of histamine's effect by cimetidine in vivo, again supporting the role of H_2 receptors. These H_2 receptors may either be those located on the tumour cells themselves or those on immunocompetent cells of the host, or both. This stimulatory effect of high local histamine concentrations may be one explanation for the importance of tumour associated mast cells. Fisher *et al*[24] have shown that the number of mast cells present in rectal cancer is prognostic – if there were more than four mast cells/high power field, survival was significantly poorer. Tumour mast cells have been shown to effect tumour cellular kinetics, with a higher proportion of PCNA positive tumour cells located close to the mast cells, suggesting a more rapid cell turnover, than tumour cells more distant.[25]

These experiments have shown that histamine has a trophic effect on at least two colorectal cell lines which can be antagonised by the H_2 antagonist, cimetidine. The response of these cell lines to histamine in vitro points to the importance of functional, tumour borne H_2 receptors.

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